BIOCHEMICAL STUDIES ON THE OCTOPUS II. PIGMENTS OF THE INTEGUMENT AND INK SACK OF OCTOPUS

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II. PIGMENTS OF THE INTEGUMENT AND INK SACK OF OCTOPUS*

By

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Introduction

Recently, Tanikawa et al (I) studied on the pigments of the integument and sepia of *Ommastrephes sloani* PACIFICUS. They reported that the integumental layer of the cuttlefish consists of four layers among which the pigmented cells exist between the 1st and 2nd layers and that the pigment of the integument belongs to the "melanin" group as well as the sepia of the ink sack, both having the same absorption maxima of 522 mμ or thereabout. Although there are no reports available on the pigments of the integument and the ink sack of octopus, the authors assumed that their pigments will probably be similar to that of the cuttlefish, considering from their classificatory kinship.

In the present report, the authors carried out the histological and spectroscopical examinations on the integumentary pigment and ink pigment of the octopus. Differing from the initial assumption, they have now the opinion that the former belongs to the "ommochrome" and the latter to the "melanin" group.

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Experimental

1. *Histological examinations of the integument of the octopus.*

The fresh integument of the octopus (*O. vulgaris* LAMARCK) was stripped off from the muscle tissue and the distribution of chromatophores in the thin integument was observed under the microscope. Since the nuclei of these chromatophores exist eccentrically and almost all the contents of the cell

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consist of pigments, these should be called "chromatophore" rather than the "pigmented cell". The states of the distribution of the chromatophores are illustrated in Figures 1 and 2. To the naked eye, three kinds of chromatophores are observed, i. e. violet black, red brown and yellow colored chromatophores. These chromatophores are distributed in the skin in mottled pattern and may be assumed to contribute to the color change of the body by their expansion or contraction. Figure 1 shows the less dense state of chromatophores (in the case of light colored) and Figure 2 shows the more dense state (in the case of dark colored).

The stereoconfiguration (Solid structure) of the integument of the octopus is illustrated in Figure 4. After fixation in 10 per cent formaldehyde solution, the integument sample was stained by the Van Gieson's dyes (Fig. 4) and another sample was stained with Haematoxylin-Eosin dyes. In the integument, the superficial layer is the epithelium and just below it, a series of chromatophores are found at the top of the subjacent thick connective tissue layer. Further deeper, a compact muscle layer runs in the direction perpendicular to the connective tissue and next, another muscle layer runs parallel to the connective tissue layer. (Cf. Figure 4) All Kinds of chromatophores are found almost in the same layer and no vertical difference of their positions in depth could be found.

The violet black chromatophores are not affected by the staining procedure above mentioned, while the other chromatophores (red brown and yellow chromatophores) are easily eliminated by the staining procedure. Table 1 shows the thickness of each layers of the integument or the size of the chromatophores.

Table 1. The thickness of the layers of the integument or the size of chromatophores.

Part	Thickness or size
Epithelium	38–50μ
Chromatophores	25–37μ × 37–50μ
Layer of chromatophores	40–50μ
Layer of connective tissue	560–750μ
First muscle tissue layer	260–340μ

2. *Extraction and purification of the pigments of the integument and ink sack.*

For the extraction of the pigment, the integument was stripped off from the muscle layer, and the integument was extracted with ethanol, acetone and ether. The red brown and yellow pigments were soluble in aqueous alcohol and acetone (80%) but insoluble in ether. Examining the residue of the integument after the extraction with these solvents microscopically, red brown

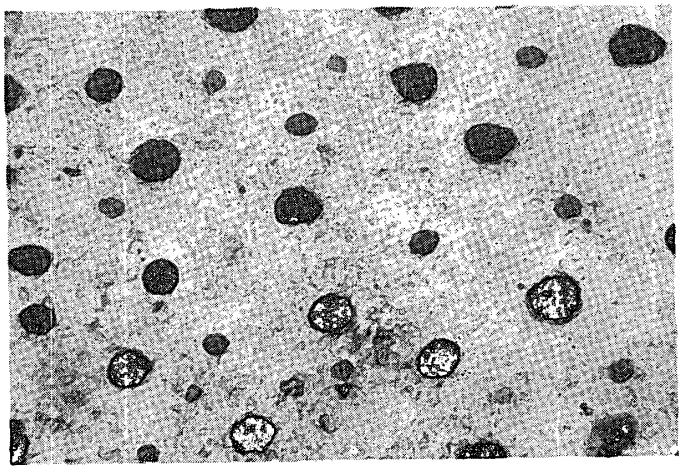


Fig. 1. Light colored integument of octopus. (×50)

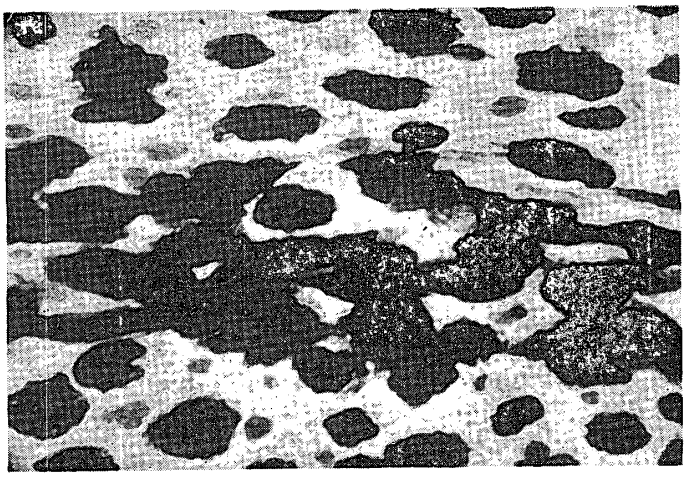


Fig. 2. Dark colored integument of octopus. (×50) aggregated chromatophores.

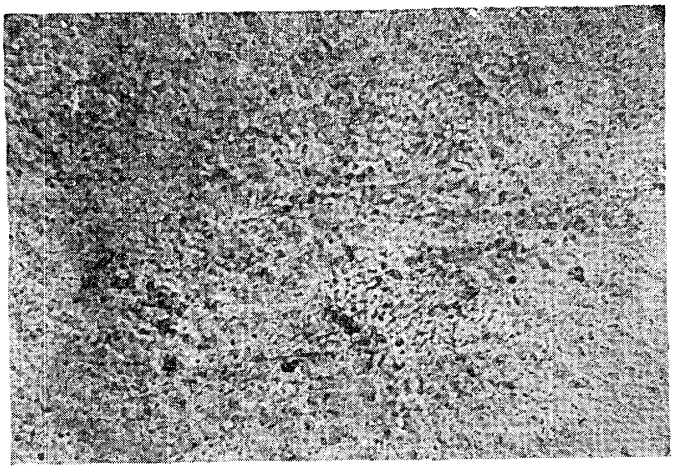


Fig. 3. Elution of chromatophores after dil. alkali treatment and reddening of tissue. (×50)

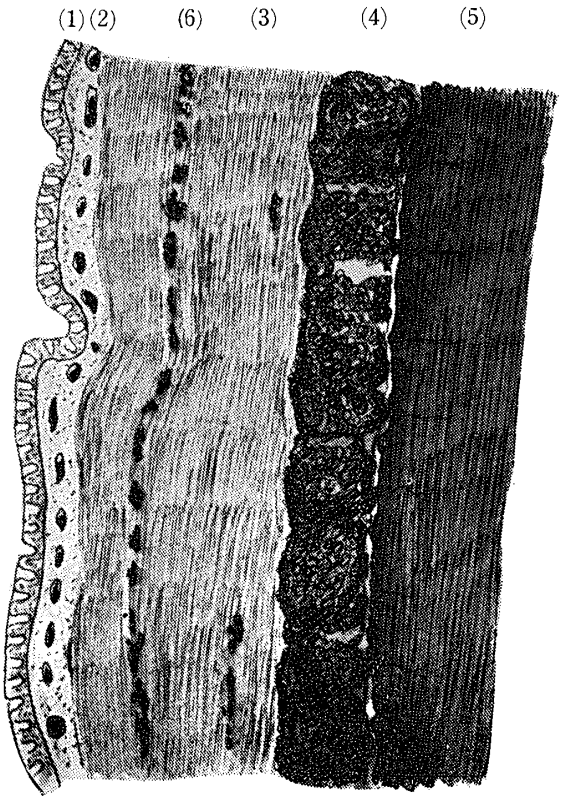


Fig. 4. Vertical section of the integument. Van Gieson's connective tissue staining.
(1) epithelium
(2) layer of chromatophores
(3) layer of connective tissue
(4) layer of muscle tissue, vertical to layer (3).
(5) layer of muscle tissue, parallel to layer (3).
(6) muscle tissue in the layer (3), vertical to layer (3).

and yellow chromatophores were eliminated, while only violet black chromatophores still remained and the integument was contracted and dehydrated. By the extraction of dehydrated integument with diluted alkali (0.1–0.05N NaOH sol.) in the thermostat at 30°C for a few hours, a clear wine red solution was obtained. All violet black chromatophores were eliminated and the residual tissue was stained pink red uniformly. The wine red solution was acidified (pH 4.2) and a pink red flocks precipitated. Then they were centrifuged and redissolved in the alkaline medium to form wine red solution. This procedure was repeated. In the later experiments, another purification method in which the dehydrated integuments are extracted with HCL-methanol (5% conc. HCL + 95% methanol) and the wine red extracts are precipitated by the addition of ether (after Schwinck) (2), was employed. In this case, dark red flocks were obtained. The ink sack of the octopus was cut off cautiously by a razor blade and the ejecting ink, free from other tissue fluids,

was collected.

On standing, the ejected ink dried to a glistening jet black solid. After the pre-treatment of the black mass with alcohol and ether, it was extracted with the diluted alkali at 30°C as before described. Differing from the case of the integument, these inks were dissolved very gradually for a longer period and a yellow brown (tan color) solution was obtained. This solution was acidified with dil. acetic acid and yellow brown pigments precipitated. This procedure was repeated and the purified product was obtained. Furthermore, human hair (female) was treated in the same way as above mentioned and hair melanin prepared was used as the control.

3. *Chemical characteristics of the pigments of the integument and ink sack*

As indicated in Table 2, the "red pigment" from the violet black chromatophores in the integument and the "yellow pigment" from the ink sack are remarkably different in their solubility towards dil. alkali. That is to say,

Table 2. Relative solubility of the "red pigment" from the violet black chromatophores and the "yellow pigment" from the ink sack towards dil. alkali.

wave length	Yellow pigment*		ratio△	Red pigment◎		ratio△
	E (3h) #	E(21.5h)		E (3h)	E(21.5h)	
360mμ	0.195	0.818	23.8%	0.765	0.970	78.8%
370	0.165	0.710	23.3	0.770	1.191	64.7
380	0.146	0.678	21.5	0.765	0.980	78.0
490	0.061	0.217	28.1	0.485	0.573	84.6
500	0.057	0.191	29.8	0.496	0.562	88.2
510	0.054	0.182	29.7	0.496	0.580	85.5

Remarks: * 0.1g of the ink was extracted in 10 ml of 0.05N NaOH in the thermostat at 30°C.
◎ Dehydrated solid of the integument (0.1g) was extracted in 10ml of 0.05N NaOH in the thermostat at 30°C.
E (3h), E (21.5h): Extinction value of the extracts after extraction for 3 hours and 21.5 hours respectively.
△ ratio: ratio of E (3h) versus E (21.5h) expressed in percentage.

Table 3. Comparison of the "red pigment" from the violet black chromatophores in the integument with the "yellow pigment" from the ink sack.

Item	Red pigment	Yellow pigment	Human hair melanin
Source	integument (epidermis)	ink sack	human hair (female)
Colour	pink red-wine red (dil. alkali)	yellow-yellow brown (dil. alkali)	Yellow-yellow brown (dil. alkali)
Spectral maxima	370–380, 500–510 mμ	general absorption decreasing from shorter to longer waves	the same as left
N %	12.56 – 15.24%	7.17 – 8.02%	
S %	13.44 – 15.45%	4.75%	

the "red pigmant" is easy soluble while the "yellow pigment" is sparingly soluble in dil. alkali. Table 3 shows the comparison of the chemical characteristics of the red and yellow pigments purified by the method described in section 2. The purified red and yellow pigments were subjected to hydrolysis by the mineral acid and then the nonpigmental part (hydrolysates) was separated from the residual pigment part. The yield of both fractions are shown in Table 4.

Table 4. Hydrolysis of the "red pigment" from the violet black chromatophores and the "yellow pigment" from the ink sack.

Item	Nonpigmental hydrolysates (protein)		Residual part (pigment)	
	Red	Yellow	Red	Yellow
Yield	88–89%* 97–98%◎	43–56% 44%	11–12% 2–3%	44–57% 56%
N %	—	—	4.28%	7.81%
S %	—	—	21.67%	2.11%
Biuret reaction	—	—	negative	negative
Ninhydrin reaction	strong positive	slight positive	—	—

Remarks: * Calculated from the weight of each fractions.
◎ Calculated from the nitrogen determination values of each fractions.

Figure 5 shows the absorption curves of the "red pigment" from the violet black chromatophores in the integument and the "yellow pigment" from the ink sack of the octopus and from the human hair. (in 0.05N NaOH solution)

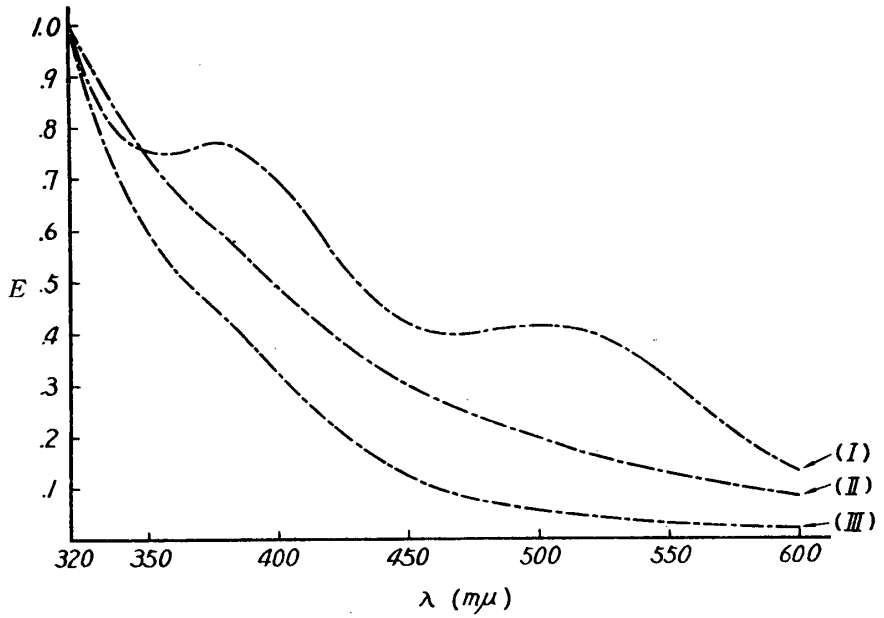


Fig. 5. (I) Red pigment from the violet black chromatophores.
(II) Yellow pigment from the ink sack.
(III) Human hair melanin.

4. *Comparison of the "red pigment" with an ommochrome after Becker.*

In 1939, Becker et al (3) proposed a new class of natural pigments, "Ommochrome", which is mainly found in the eye pigment of insects. The authors compared the "red pigment" from the violet black chromatophores with an ommochrome (skotommin after Becker). The results are given in Table 5. These characteristics listed in Table 5 shows that both pigments are almost similar to each other but differ from the melanin.

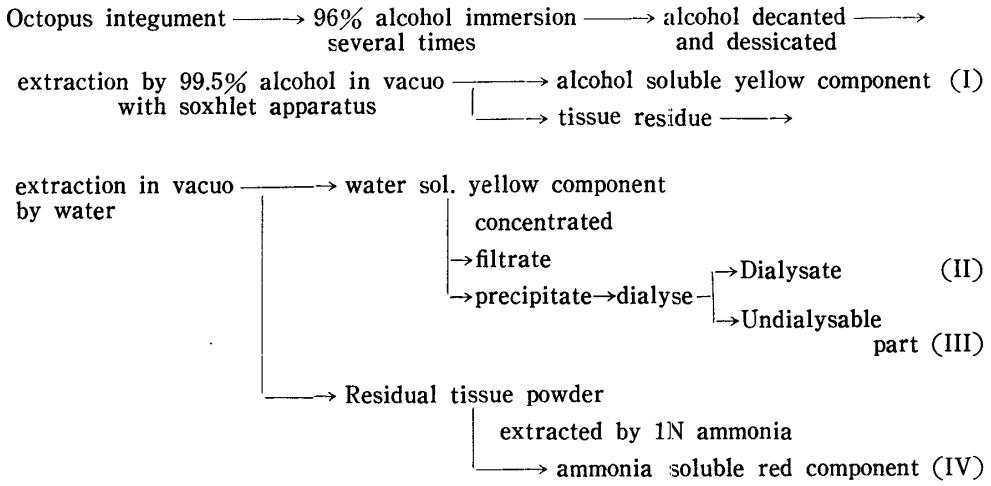
Table 5. Comparison of the "red pigment" from the violet black chromatophores with an ommochrome.

Items	Red pigment from the violet black chromatophores	Ommochrome (skotommin) after Becker
Dialysability	undialysable, but trace is dialysable when the pigment was dissolved	undialysable, but trace is dialysed in alkaline solution
Solubility		
Ammonia	sol. (blood red color, sensitive)	sol. (red color, sensitive)
NaOH	sol. (winered color, sensitive)	sol. (red, sensitive)
Conc. HCL	sol. (brown with violet tint)	sol. (brown with violet tint)
Conc. H_2SO_4	sol. (violet, Halochromy)	sol. (violet, Halochromy)
HCOOH	sol. (deep red)	sol. (deep red)
HCL-acidic alcohol or methanol	sol. (deep red)	sol. (deep red)
Boiling phenol	sol.	sol.
Glacial acetic acid	insol.	insol.
Dil. mineral acid	insol.	insol.
Fat solvent	insol.	insol.
Redox reaction Oxidation		
HNO_3 (1 : 1)	degrade to yellow product	degrade to yellow product
Alkaline H_2O_2	decolorize to yellow	decolorize to yellow (in the presence of trace iron)
Na Nitrite in acetic acid	decolorize to yellow	decolorize to yellow
Reduction		
Hyposulphite	reduced to deep red	reduced to deep red
Sulphur dioxide	reduced to red	reduced to red
Hyposulphite, ammonium sulphide, and zinc dust in alkaline sol.	not reduced	not reduced
Spectral maxima	370–380, 500–510 mμ (reduced stage) 450 mμ (oxidized stage, shoulder like maxima)	480, 510 mμ weak maxima and 405, 440 mμ more weaker maxima (Becker) 500–510 mμ (reduced stage, Schwink) 450 mμ (oxidized stage, Schwinck)

5. *Fluorescent component of the octopus integument.*

Since the authors found the fluorescent component in the integument of the octopus, the content of the total fluorescent component in the integument was determined after Yagi's method of flavines (4), employing riboflavine as a standard. The content of the fluorescent component was 1.7~2.0 microgram per 1 g wet integument as riboflavine, however the color of its fluorescence differs from that of riboflavine. Then the integument of the octopus was fractionated after Becker-Schopf's scheme (3) and the fluorescent component of each fractions were examined under the ultra violet lamp.

Becker-Schopf's scheme



The results are shown in Table 6.

Table 6. Fluorescence of each fractions in the integument of the octopus fractionated after Becker-Schopf's scheme.

Fraction	Octopus integument		Ommochrome after Becker	
(I)	sky blue			
(II)				
Acetic acid acidic	sky blue-violet	#	blue violet	*
Soda alkaline	sky blue	#	green blue	*
Neutral	sky blue	#	sky blue	*
(III)				
Acetic acid acidic	yellow green with violet tint		bright violet	○
Soda alkaline	yellow green		sky blue	○
NaOH alkaline	yellow green		grey blue	○
(IV)	almost none		almost none	◎

Remarks : * The fluorescence of this fraction resembles chrysopterin. (Becker)
○ The fluorescence of this fraction is due to xanthommin. (Becker)
◎ This fraction is skotommin. (Becker)
The fluorescence of this fraction is strongest.

Discussion

The pigment of the ink sack of the octopus is identical with the human hair melanin considering from the spectral properties and solubility. Oxidized melanin (black) is fairly stable and is gradually reduced in dil. alkaline medium to form a yellow (or tan color) solution. These yellow melanins, reduced form of melanin according to Lerner and Fitzpatrick (5), show the characteristic absorption curve decreasing from shorter to longer waves without peak (general absorption). Absorption maxima at 522 mμ or thereabout according to Tanikawa (1) could not be found in the case of yellow melanins from the ink sack of the octopus or from the human hair. Relative solubility towards dil. alkali and the other properties (Tables 2, 3 and 4) are clearly different from the pigment of the integument.

In the integument of the octopus, violet black, red brown and yellow chromatophores are distributed under the epithelium and the vertical difference of their positions in depth could not be found. The red brown and yellow pigments are fairly soluble in aqueous alcohol and acetone (80%) and insoluble in ether. These pigments are eliminated by the usual staining procedure while the violet black pigment still remains after the staining and are only extractable with dil. alkali or HCL-methanol.

When the octopus is boiled in water, pH of the medium shifts to the more alkaline side as the result of dissolution of nitrogeneous basic compounds (cf. Table 7) and this facilitates the elution of wine red pigment from the violet black chrometophores (ommochrome) easier and the wine red pigment eluted stains the tissue protein (collagen) red uniformly. (figure 3). This is the reason why the octopus turns red when boiled in water.

Table 7. Changes of pH of the medium in which octopus was boiled.

medium	before boiling	after boiling
water	6.2	6.8
salted water	6.2	7.0
dil. alkali	9.2	9.6

The wine red pigment from the violet black chromatophores in the integument has two absorption maxima at 370~380 mμ and 500~510 mμ (in the reduced stage) and only one weak maximum (shoulder like) at 450 mμ (in the oxidized stage). This pigment is very similar to the "ommochrome" (skotommin after Becker) as indicated in Table 5. Ommochromes were first discovered by Becker chiefly in the eye pigment of insects and afterwards Schwinck (2) found them in the skin pigment of *Sepia officinalis*. Characteristics for ommochrome are recognized in the solubility, the redox reaction, the precipitability

and the spectral properties. In the case of the octopus, from the various similarities, the authors have the opinion that the integumentary pigments belong to the ommochrome group and not to the melanin group. According to Becker, ommochrome are classified into two groups, i. e. "ommine" of high molecular weight and "ommatine" of low melecular weight.

Comparison of melanin with ommochrome

	precursor	color of		formation
		reduced form	oxidized form	
melanin	Indole 5,6-quinone	yellow (tan)	black	tyrosine, dopa metabolism
ommochrome (ommine and ommatine)	3-oxykynurenine	red	yellow	tryptophane, kynurenine metabolism

However, Butenandt et al (6) recently reported on the isolation of four ommatines (xanthommatine, hydro-xanthommatine, rhodommatine and ommatine C) in the secretion of the butterfly, *Vanessa urticae*. They pointed out that xanthommatine and rhodommatine have in part the group properties of "ommatines" reported by Becker respectively and that the Becker's ommatine is not of uniformity but the mixture of xanthommatine, rhodommatine and a colorless product. Therefore, the precise determination of the grouping about our ommochrome of the octopus integument must be reexamined.

In 1943, Fox and Updegraff(7) reported on the occurrence of "Adenochrome", a glandular pigment in the branchial heart of the two spotted octopus, *Paroctopus bimaculatus*. This pigment also resembles our ommochrome in the integument in many respects, for example, its spectral properties (wine red color solution in dil. alkali with absorption maximum at 505 mμ), and its solubility (insoluble for fat solvent and very soluble for alkalis). At present, the authors have not yet examined in detail the comarison of the adenochrome with our ommochrome in the integument, therefore, the identity of the adenochrome with our pigment should be reserved for another opportunity.

Melanin in the ink sack and the ommochrome in the integument together combine with the tissue protein very tightly in the natural state and well accompany with each other in the case of purification process, but the protein is not an essential part for the pigments unlike the conjugated chromoprotein. Fluorescence of the water soluble yellow component of the octopus integument somewhat differs from that of xanthommin after Becker and is clearly different from those of riboflavine and ichthyopterine (fluorescyanine).

Summary

(1) The ink pigment in the ink sack of the octopus, *Octopus vulgaris* LAMARCK, belongs to the melanin group as well as the human hair melanin,

considering from the solubility, spectral properties and the other analytical values.

(2) In the integument of the octopus, violet black, red brown (orange) and yellow chromatophores are found. The histological structure was described.

(3) Red pigment from the violet black chromatophores in the integument, dominant component of the skin pigments, belongs to the "ommochrome" of Becker and not to the melanin group. When the octopus is boiled in water, eluted red ommochrome stain the tissue protein (collagen) red uniformly. The red pigments (the reduced form) are concentrated and polymerized in the chromatophore to form violet black color or oxidized to form yellow color.

(4) Not only the expansion and the contraction of the chromatophores, but also the redox reaction of the pigment itself as the hydrogen donor or accepter, contribute to the color change of the octopus.

References

1) Tanikawa, E., Akiba, M. (1952). Bull. Fac. Fish. Hokkaido Univ. **13**, No. 1 81–94.
2) Schwinck, I. (1953). Naturwiss. **13**, 365.
3) Becker, E. (1939). Zent. bl. Biol, **59**, 597–627.
ibid (1941). Naturwiss. **8**, 237.
4) Yagi, K. (1951). J. Biochemistry **38**, No. 2, 16.
5) Lerner, A. B., Fitzpatrick, T. B. (1953). Pigment cell growth, Academic press, New York 321 pp.
6) Butenandt. A. et al (1954). Ann. 586, 3.
7) Fox, Updegraff (1943). Arch. Biochem. I. 339–356.